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Imidazoline binding sites in human placenta: evidence for heterogeneity and a search for physiological function

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1 An α_2-adrenoceptor antagonist, idazoxan, that binds to both α_2-adrenoceptors and to imidazoline sites (IR), has been used to characterize human placental IR. Human placenta is shown to be the richest source of IR (1800 ± 100 fmol mg^{-1} protein; K_d 38.9 ± 3.4 nM).

2 Primary cells derived from human placenta and grown in monolayers, also displayed a high density of receptors (3209 ± 136 fmol mg^{-1} in cytotrophoblasts and 3642 ± 144 fmol mg^{-1} protein in syncytiotrophoblast enriched cell culture).

3 [^{3}H]-idazoxan did not show binding characteristics of α_2-adrenoceptors in human placental membranes or human trophoblastic cells, thus making it a ligand of choice to study the imidazoline site. The tissue appeared to be lacking α_2-adrenoceptors in that other α_2-adrenoceptor ligands, [^{3}H]-rauwolscine and [^{3}H]-clonidine, do not bind to α_2-adrenoceptors in human placenta.

4 IRs are localized on the cell surface, as determined by the release of bound [^{3}H]-idazoxan from cells, when washed with high ionic/acidic medium.

5 Imidazoline receptors of human placenta display high affinity for amiloride (72 ± 27 nM). The high affinity was used as a criterion to classify IR to IR_a subtype (placenta, rabbit kidney, rabbit liver and rabbit adipose cells) as opposed to the IR_b subtype which display low affinity for amiloride (>2 μM, in all the other tissues).

6 Several novel ligands comprising a guanido functional group attached to an aromatic residue (e.g. benziliden-amino-guanidine (BAG), guanido pyrole) display pronounced selectivity for IR over the α_2-adrenoceptors as the affinity of BAG is about 40 fold higher ($K_d = 18.9 \pm 13.8$ nM in human placenta), than the affinity for α_2-adrenoceptors ($K_d = 768 \pm 299$ nM in human platelets). Imidazoline sites bind selectively BAG and other guanido ligands thus indicating a distinct structural requirement at its site of binding.

7 K^+ channel blockers and monovalent ions (e.g. Cs^+ and NH_4^+) interfere with idazoxan binding to IR, indicating a possible involvement of IR in K^+ transport.

Keywords: Human placenta; imidazoline sites; CDS-clonidine displacing substance

Introduction

Imidazoline binding sites were recently described as pharmacologically distinct from α_2-adrenoceptors. These sites were identified and characterized in rabbit kidney cells (Coupry *et al.*, 1987; 1989; Hamilton *et al.*, 1988), pig kidney (Vigne *et al.*, 1989), fat cells (Langin & Lafontan, 1989), rat liver and lung, human platelets (Zonnenschein *et al.*, 1990), rat medulla oblongata (Ernsberger *et al.*, 1987) and guinea-pig ileum (Kupitz, Diamant & Atlas, unpublished observations).

These sites are not a subtype of adrenoceptors since (a) they can be separated from the α_2-adrenoceptors on lectin-affinity column (Parini *et al.*, 1989), (b) they do not recognize adrenaline nor the specific α_1- or α_2-adrenoceptor antagonists such as prazosin and yohimbine (see Atlas, 1991). However, the site binds, with very high affinity, α_2-adrenoceptor ligands which have guanidine and imidazoline structures (Coupry *et al.*, 1987; Langin & Lafontan, 1988; Michel *et al.*, 1989; Wikberg, 1989; Zonnenschein *et al.*, 1990). The density of the imidazoline sites dramatically exceeds the density of α_2-adrenoceptors in most of the cells where both are present, and appear in high density in cells which lack α_2-adrenoceptors such as rat liver (Zonnenschein *et al.*, 1990). Although a large variety of ligands bind with high affinity to the imidazoline sites and the sites are found in many cells, so far no function has been attributed to them.

A possible interaction of the imidazoline receptors with K^+-channels was suggested, based on the reduction in [^{3}H]-idazoxan specific binding produced by a high K^+ concentration in kidney cells (Coupry *et al.*, 1987) and by K^+-channel blockers in liver cells (Zonnenschein *et al.*, 1990).

Furthermore, in rat isolated kidney cells, structures with the imidazoline moiety induce inhibition of ^{22}Na uptake and changes in intracellular pH (Bidet *et al.*, 1990). Since the effects correlate with the binding order potency of imidazoline ligands, it was suggested that they are mediated by imidazoline receptors.

In this paper we have characterized imidazoline binding sites in human placenta, where their density is the highest reported so far. In addition, in human placental membranes, no α_2-adrenoceptors were detected with the radiolabelled specific α_2-adrenoceptor antagonist, [^{3}H]-rauwolscine, or the α_2-adrenoceptor agonist, [^{3}H]-clonidine. Furthermore no specific binding to α_2-adrenoceptors was observed with [^{3}H]-idazoxan.

We have used (a) placental membrane preparation, (b) freshly isolated cytotrophoblasts and (c) cultured cytotrophoblasts (which fuse to form syncytia *in vitro*) for the pharmacological characterization of imidazoline receptors and for exploring the physiological function of these sites.

Methods

Placental membrane preparation

Placental membranes were prepared from human full term placentas obtained from normal pregnancies immediately

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after vaginal delivery, and processed as previously described (Gradey-Levassort *et al.*, 1984).

Isolation of trophoblast

Cytotrophoblast cells were prepared from human term placentas and cultured as previously described (Kliman *et al.*, 1986; Gileadi *et al.*, 1988). The cells, plated in multiwell dishes (0.5 to 1.0 million cells per well), gradually fused to form syncytia over a period of 24 to 72 h and were grown in culture up to 4 days.

Preparation of brush border membrane vesicles

Microvillous membranes were isolated from human term placentas as previously described (Johnson & Smith, 1988).

Platelet membrane preparation

Platelet membranes were prepared as previously described (Diamant *et al.*, 1987).

Protein determination

Protein was determined according to the method of Lowry *et al.* (1951), with bovine serum albumin used as a standard.

Binding of radioligands to placental membranes

The binding assay was carried out essentially as described for liver membranes (Zonnenschein *et al.*, 1990). Imidazoline receptor and α_2-adrenoceptors were characterized using cirazoline and noradrenaline respectively, for determining non specific binding. In saturation experiments the K_d and B_{max} values were analyzed with Enzfitter, a non linear regression data analysis programme (Leatherbarrow, 1987). Affinities of various ligands, were determined in displacement assays, using 3–4 nM [^{3}H]-idazoxan. The K_i values were calculated using EBDA- LIGAND computerized programme (Munson & Rodbard, 1980).

Binding of [^{3}H]-idazoxan to trophoblast cells

Freshly prepared trophoblast cells in suspension or plated in a 24 multiwell dish (NUNC) (5×10^5 cells/well) were washed with isotonic KRH buffer pH 7.4 (composition, mM): NaCl 108, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES-Tris 20, glucose 10, and incubated with increasing concentrations (2 to 30 nM) of [^{3}H]-idazoxan in the presence and in the absence of 20 μM cirazoline. Incubation of the cells was carried out for 4 h at 4°C. The suspended cells were filtered through GF/C filters, washed with cold isotonic buffer and processed as described for membranes (Zonnenschein *et al.*, 1990). Plated cells were washed 3 times with cold isotonic buffer, dissolved in 0.1% SDS and counted in a scintillation counter.

Displacement of [^{3}H]-rauwolscine from human platelet membranes

The assay was performed as previously described (Diamant *et al.*, 1987); 4–5 nM [^{3}H]-rauwolscine, and 50 μM adrenaline were used for evaluation of non specific binding.

Binding of [^{3}H]-idazoxan and [^{3}H]-dihydroalprenolol to membrane vesicles

[^{3}H]-idazoxan binding to vesicle membranes was carried out in 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$, at 4°C for 4 h as described for cytotrophoblast cells. Specific binding to IR and α_2-adrenoceptors was determined with 20 μM cirazoline and 50 μM adrenaline respectively. [^{3}H]-dihydroalprenolol binding was determined at 30°C for 40 min using 10 μM propranolol for evaluation of non specific binding.

Alkaline phosphatase activity

Placental alkaline phosphatase activity was determined at pH 10.5 with p-nitrophenyl phosphate as the substrate according to Galski *et al.* (1981).

ATPase activity

ATPase activity was determined by colorimetric measurement of inorganic phosphate (Pi) released from ATP during incubation at 37°C (Fiske & Subbarow, 1925). The assay conditions were according to Post & Sen (1967).

Na^+/K^+ ATPase activity was dependent on Na^+ and K^+ ions and inhibited by ouabain (0.1 mM). Mg-ATPase activity was absolutely dependent on Mg^{2+} ions.

Purification of clonidine displacing substance (CDS)

CDS was purified according to a procedure previously described (Atlas & Burstein, 1984; Atlas *et al.*, 1987).

Materials

[^{3}H]-idazoxan and [^{3}H]-rauwolscine were obtained from the Radiochemical Centre, Amersham (England); [^{3}H]-clonidine and [^{14}C]-aminoisobutyric acid ([^{14}C]-AIB) from NEN Research Products (U.S.A.). Clonidine and amiloride were purchased from Sigma (U.S.A.). The following were gifts: rilmenidine (53341) from Servier, France; naphazoline and UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) from Lilly Research Laboratories (U.S.A.); guanoxan and 94/2007.B ([4-(chloromethyl)-2-thiazolyl]guanidine) from Dr A.L. Green, SmithKline & Beecham (England); cirazoline from Synthelabo (France); amiloride analogues (5-(N-ethyl-N-isopropyl) amiloride (EIPA); 5-(N,N-hexamethylene) amiloride (HMA); 5-[N-methyl-N-(guanidinocarbonylmethyl)] amiloride-(MGCMA) from E.J. Cragoe; idazoxan (RX 781094) from Reckitt & Colman (U.K.); guanabenz was synthesized by D.A. (Diamant & Atlas, 1986); guanidopyrol and guanidotyramine were synthesized by D.A. and benzyliden-aminoguanidine (BAG) was from A. Dagan (Makor Chemicals, Israel).

Results

Binding of [^{3}H]-idazoxan to placental membrane

[^{3}H]-idazoxan was added in increasing concentrations (0.4 to 30 nM) to placental membranes in the absence and presence of cirazoline (20 μM) and incubated at 4°C for 4 h. Saturation binding isotherms obtained for [^{3}H]-idazoxan (Figure 1) gave a $B_{max} = 1800$ fmol mg^{-1} protein whereas non-specific binding was 10% to 15% of the total binding, and a $K_d = 38.9 \pm 3.4$ nM. The dissociation constant value is higher than the K_d values reported for liver or kidney membranes (10 and 1.5 nM respectively, Zonnenschein *et al.*, 1990). The K_d value for idazoxan was obtained by extrapolation from a non-linear regression data analysis using the computerized Enzfitter programme (Leatherbarrow, 1987). The time course of [^{3}H]-idazoxan (4 nM) binding to placental membrane at 4°C and at 30°C shows different saturation values (155 and 60 fmol mg^{-1} protein respectively) but equilibrium is reached after 30 min at both temperatures (Figure 2). Interestingly, in liver membranes, maximal binding (at 4 nM [^{3}H]-idazoxan) is similar at 4°C and 30°C but equilibrium is reached faster at 30°C compared to 4°C (Figure 3). [^{3}H]-idazoxan was not displaced by 100 μM adrenaline (or noradrenaline) indicating that it does not label α_2-adrenoceptors in placental membranes (Figure 4). Furthermore, binding of [^{3}H]-rauwolscine (0.3 to 14 nM) or [^{3}H]-clonidine (0.5 to 20 nM) to human placental membrane in the absence and in the presence of

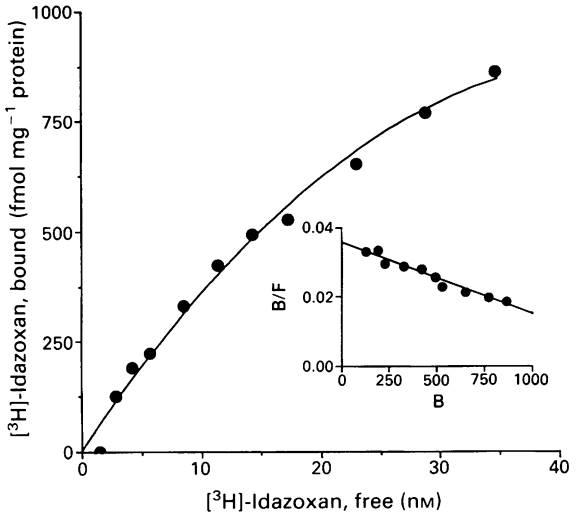

Figure 1 Specific binding of [^{3}H]-idazoxan to human placental membranes. The assay was carried out at 4°C for 4 h and non specific binding was determined in the presence of 20 μM cirazoline. A Scatchard plot (presented in insert) was derived from non-linear analysis of the data, using the Enzfitter computer curve fitting programme (Leatherbarrow, 1987). B represents specific bound [^{3}H]-idazoxan in fmol mg^{-1} protein; F free [^{3}H]-idazoxan concentration in nM. The data presented are a representative experiment, which was repeated 8 times with similar results (see Table 4).

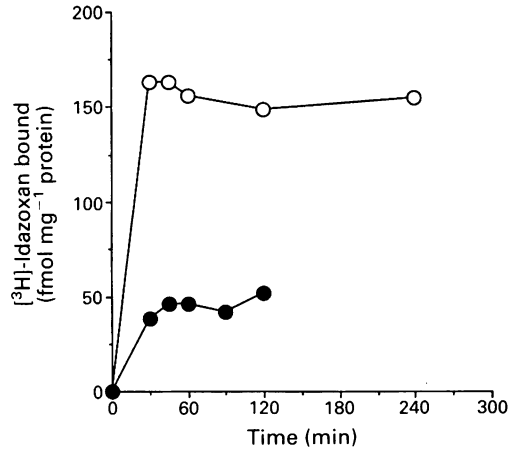

Figure 2 Effect of temperature on [^{3}H]-idazoxan specific binding to human placental membranes. Binding assay was carried out using 4 nM [^{3}H]-idazoxan in 250 μl of 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 150 μg membranal protein at 4°C (○) and 30°C (●). For each time interval non specific binding was determined using 20 μM cirazoline. The data represent a typical experiment ($n = 7$).

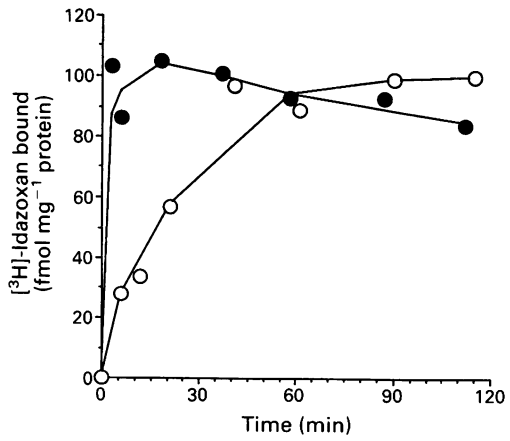

Figure 3 Effect of temperature on [^{3}H]-idazoxan specific binding to rat liver membranes. The assay conditions were as indicated in Figure 2: incubations at 4°C (○) and 30°C (●). The data represent a typical experiment ($n = 2$).

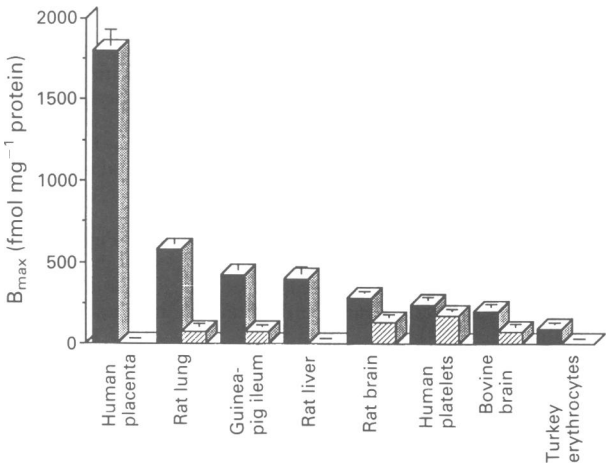

Figure 4 Specific binding of [^{3}H]-idazoxan to membranes of different tissues. Human placenta and platelet membranes were prepared as described in the methods section; rat lung and liver (Zonnenschein *et al.*, 1990) rat and bovine brain membranes (Atlas & Burstein, 1984); the guinea-pig ileum membranes (Kupitz, Diamant & Atlas, unpublished), turkey erythrocytes membrane according to Feder *et al.* (1986). Membranes (150 to 250 μg protein) were incubated with increasing concentrations (0.5 to 30 nM) of [^{3}H]-idazoxan in 250 μl of 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$. The incubation was carried out at 30°C for 40 min except for human placental membranes which were incubated at 4°C for 4 h. Non specific binding was determined with 20 to 100 μM cirazoline (solid columns) and adrenaline 100 μM (hatched columns). B_{max} values were analyzed using the Enzfitter computer programme (Leatherbarrow, 1987).

100 μM adrenaline failed to show any specific binding, thus indicating either the absence of α_2-adrenoceptors or a very low affinity of these ligands for α_2-adrenoceptors compared with other tissues (data not shown). As shown in Figure 4, human placental membranes display the highest number of IR receptors compared to IR sites in different tissues. Like rat liver membranes, placental membranes do not express α_2-adrenoceptors (see above).

Displacement of the [^{3}H]-idazoxan bound to membranes by various imidazoline and guanidine ligands and CDS

Displacement experiments were carried out with crude placental membranes. The guanidine and the imidazoline ligands, some of which are used as antihypertensive drugs, have a common structural feature; namely, a guanidine or an imidazoline group. As shown in Table 1, the guanidine-type ligands displayed high affinity towards the imidazole receptor, with guanoxan, BAG (analogue of guanabenz lacking two substituted chloride on the benzyl ring) and guanidopyrol being most effective (K_is 3.55, 18.9 and 11.2 nM respectively). Both guanidopyrol and BAG have been shown to bind with relatively low affinity to α_2-adrenoceptors (see Table 1), thus making them selective drugs for IR. Of the imidazoline type ligands, cirazoline displayed the highest affinity for IR whereas UK 14,304 and clonidine displayed poor affinity for IR with higher affinity for α_2-adrenoceptors which makes them specific α_2-adrenoceptor ligands. Clonidine displacing substance (CDS), extracted from bovine brain and purified according to Atlas & Burstein (1984) and Atlas *et al.* (1987), displaced [^{3}H]-idazoxan specifically bound to placental membranes with an apparent K_d of 2.32 units (Table 1).

Table 1 Affinity of different ligands to imidazoline sites (IR) and α_2-adrenoceptors (α_2 AR) in placental and platelet membranes

	K_i (nM)		
Ligand	*IR placenta*	*α_2AR platelets*	*IR/α_2AR affinity*
Guanoxan	3.55 ± 2.91	85.0 ± 11.0	23.94
Guanidopyrol	11.2 ± 6.3	10100 ± 1515	905.00
BAG	18.9 ± 13.8	768.0 ± 299	40.63
Cirazoline	38.6 ± 12.2	142.1 ± 24.5	3.68
Idazoxan*	38.9 ± 3.4	3.94 ± 0.25	0.10
Guanabenz	48.9 ± 13.7	23.9 ± 8.2	0.49
Guanidotyramine	105.0 ± 38.0	4640 ± 2366	44.19
Naphazoline	128.0 ± 57.1	38.85 ± 22.8	0.30
94/2007B	673 ± 114	81500 ± 65200	121.10
UK-14304	1747 ± 227	18.8 ± 4.7	0.01
Clonidine	7390 ± 4367	36.1 ± 8.0	0.005
Rilmenidine	11470 ± 2010	144.6 ± 37.3	0.013
Amiloride	72.2 ± 26.6	35037 ± 13384	486.6
Benzamil	> 10,000	2268 ± 1295	> 0.3
CDS**	2.32 Unit	1.28 Unit	0.55

Membranes (150 to 250 µg protein per assay) were incubated with 3–4 nM [^{3}H]-idazoxan or with 4–5 nM [^{3}H]-rauwolscine, in 250 µl of 50 mM Tris HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and appropriate concentration of ligands. For placenta the incubation was performed at 4°C for 2 h and non specific binding was determined with 20 µM cirazoline. For platelet membranes, the assay was performed for 40 min at 30°C and 50 µM adrenaline was used for non-specific binding. K_i values were calculated by EBDA-LIGAND analysis using: K_d of idazoxan in placental membranes = 39 nM, and K_d of rauwolscine = 4.3 nM for platelet membranes.
*The value represents K_d (see Scatchard analysis in Figure 1).
**A unit of amount of CDS (clonidine displacing substance) was defined as the activity needed to displace 50% of [^{3}H]-clonidine specifically bound to rat brain membranes (Atlas & Burstein, 1984).

Table 2 Effect of K^+-channel blockers and monovalent cations on [^{3}H]-idazoxan binding in placental membranes

	IC_{50} (mM)
4-Aminopyridine	0.025 ± 0.01
CsCl	2.63 ± 0.76
NH_4HCO_3	3.03 ± 2.86
NaCl	29.6 ± 11.70
KCl	39.5 ± 0.20

The assay was performed as described in Table 1 for placental membranes.

Table 3 Binding of [^{3}H]-idazoxan to human placental membranes and to trophoblast cells

	B_{max} (fmol mg^{-1} protein)	*K_d (nM)*
Term placental membranes	1800 ± 100	38.9 ± 3.4 (n = 8)
Cytotrophoblasts	3209 ± 136	15.8 ± 1.4 (n = 5)
Cultured trophoblasts	3642 ± 144	36.3 ± 3.0 (n = 5)

Freshly prepared cytotrophoblast cells in suspension or plated in monolayer and grown in culture for 24 to 96 h, were incubated with 2 to 30 nM [^{3}H]-idazoxan (in presence and absence of 100 µM cirazoline) for 4 h at 4°C. The binding assay was terminated by rapid filtration through GF/C filters (for suspended cells) or by washes with cold isotonic buffer (for plated cells). B_{max} and K_d values were calculated using Enzfitter-non linear regression data analysis programme (Leatherbarrow, 1987). The data are average ± s.e.mean of 8 experiments for placental membranes, of 3 experiments for cytotrophoblasts and of 5 experiments – for cultured trophoblasts.

Displacement of [^{3}H]-idazoxan by amiloride and its analogues

Amiloride, an inhibitor of the Na^+/H^+ antiporter, which has a guanido structure, binds with high affinity to IR, K_i = 72 ± 27 nM (as determined by displacement of [^{3}H]-idazoxan from crude placental membranes), and very poorly to human platelet membranes, K_i = 35 µM (Table 1). Amiloride analogues, substituted on the 5-amino nitrogen of the pyrazine nucleus, displayed lower affinity towards imidazoline sites but slightly higher affinity towards α_2-adrenoceptors (data not shown). Benzamil, an amiloride analogue, with a benzyl group substituted on the nitrogen of the guanido group (Kleyman & Cragoe, 1988) displayed no affinity toward IR receptors, indicating the importance of an intact guanidino group for recognition (Table 1).

Effect of monovalent ions and K^+-channel blockers on binding of [^{3}H]-idazoxan to placental membranes

The order of potency of the various ions in displacing [^{3}H]-idazoxan was $Cs^+ > NH_4^+ > Na^+ > K^+$. 4-Amino-pyridine (4-AP), a K^+ channel blocker, was very effective in displacing idazoxan from placental membranes (IC_{50} = 25 µM, Table 2), as compared to its potency in inhibiting K^+ channels in neuronal cells.

Binding of [^{3}H]-idazoxan to trophoblasts in suspension or grown in culture

Binding to cells in suspension or grown in monolayers was carried out for 4 h at 4°C. As shown in Table 3, freshly prepared cytotrophoblast cells show a higher number of IR sites (B_{max} = 3209 ± 136 fmol mg^{-1} protein) than that observed for human placental membranes and the K_d value for idazoxan was lower (K_d = 15.8 ± 1.3 nM, Figure 5).

B_{max} values were similar when trophoblasts were kept in culture for different time intervals (24 to 96 h). The affinity for idazoxan in cultured cells was similar to the affinity observed for placental membranes.

Binding of [^{3}H]-idazoxan to plasma membrane or to intracellular compartments

To examine the possibility that [^{3}H]-idazoxan binds intracellularly (Tesson *et al.*, 1991), the cells were washed with 0.2 M acetic acid/0.5 M NaCl at the end of the binding assay and cell viability was verified by Trypan blue staining (Haigler *et al.*, 1980; Mahan *et al.*, 1985). The acidic-hypertonic wash of the cells was shown to remove all the ligand bound at the cell surface leaving the cells with the ligand inside, intact. As shown in Table 4, 86.5% of the specific binding was released from the cells under these conditions, indicating that it was associated with the cell surface.

Effect of temperature on [^{3}H]-idazoxan binding to placental cells

In trophoblast cells, as in placental membranes, the number of [^{3}H]-idazoxan (2.5 nM) sites labelled at 4°C are twice the sites labelled at 30°C (Figure 6). Possible internalization and binding to inner organelle membranes at 30°C was examined by applying the method of Haigler *et al.* (1990). At the end of 2 h binding, the cells were washed with 0.2 M acetic acid/0.5 M NaCl. The wash out and the washed cells were counted. As shown in Figure 6 (insert) binding was washed out completely, and no binding was found inside the cells.

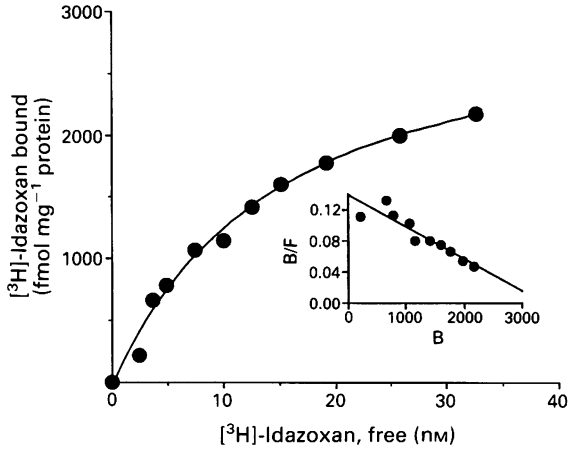

Figure 5 Specific binding of [^{3}H]-idazoxan to human cytotrophoblast cells. Binding assay was performed on cells, suspended in isotonic KRH buffer (see Methods), containing 2–30 nM [^{3}H]-idazoxan, in the presence and in the absence of 20 μM cirazoline. The assay was carried out for 4 h at 4°C. A Scatchard plot (presented in insert) was derived from non linear analysis of the data using Enzfitter computer programme (Leatherbarrow, 1987). The data presented are of a representative experiment which was repeated 3 times.

Table 4 Distribution of [^{3}H]-idazoxan binding in trophoblast cells

	B_{max} (fmol mg^{-1} protein)	B_{max} (%)	K_d (nM)
Surface	4135 ± 785	86.5	38.5 ± 11.3
Inside	647 ± 146	13.5	27.7 ± 9.7

Trophoblast cells, grown for 24 h in culture, were incubated with 1–22 nM [^{3}H]-idazoxan at 4°C. At the end of 4 h incubation, the cells were washed with acidic high salt buffer (0.5 M NaCl/0.2 M acetic acid, pH 2.5) and the radioactivity in the washout and the cells was measured.

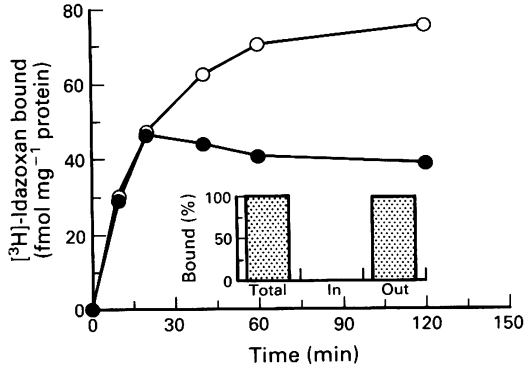

Figure 6 Effect of temperature on [^{3}H]-idazoxan specific binding to human trophoblast cells. Trophoblast cells, grown in culture for 24 h, were incubated with 2.5 nM [^{3}H]-idazoxan at 4°C (○) and 30°C (●) in the absence and in the presence of 20 μM cirazoline. At the end of incubation period the cells were washed with isotonic buffer, solubilized in 0.1 N NaOH and counted. Insert: cells were incubated for 2 h with [^{3}H]-idazoxan at 30°C (in the presence and in the absence of 20 μM cirazoline), then incubated with 0.2 M acetic acid/0.5 M NaCl pH 2.5 for 15 min, the medium removed and counted ('Out') and the remaining radioactivity of the cells was counted ('In'). 'Total' = the radioactivity observed with regular isotonic buffer wash (100%).

Localization of imidazoline receptor

Determination of [^{3}H]-idazoxan binding activity in brush border membranes and crude membranes (enriched in basolateral membranes as determined by distribution of the activity of alkaline phosphatase and Mg-ATPase), revealed that only 8% of the binding sites were present in the brush border membrane and 92% of the idazoxan binding sites were localized in the basal membrane and other cellular membrane structures (Table 5). Imidazoline receptors co-localized with β-adrenoceptors in that 85% of [^{3}H]-dihydroalprenolol binding sites were found in basolateral membrane enriched vesicles and only 15% in brush border membranes. On the other hand α_2-adrenoceptors were present in a very small number in the brush border membrane fraction (63 fmol mg^{-1} protein) and were absent from the crude membranes preparation.

ATPase activity

The driving force for many transport processes in placenta is generated by ATPase activities. We determined various ATPase activities in crude placental membranes enriched with basolateral membranes and in isolated brush border membranes. High activity of Mg-ATPase was found in brush border membranes, two times higher than in basolateral membranes enriched preparation (as expected from asymmetric transporting epithelial cells Table 5). This activity was not affected by any of the high affinity guanido and imidazoline ligands (Table 6). No Na^+/K^+ dependent ouabain-sensitive ATPase activity could be detected, most likely due to the very high Mg-ATPase activity.

In bovine brain, where Na^+/K^+ ATPase activity could be measured, none of the imidazoline/guanido-ligands tested had a significant effect on Na^+/K^+ ATPase activity (Table 6).

Discussion

A new population of binding sites labelled with [^{3}H]-idazoxan, which is pharmacologically distinct from α_2-adrenoceptors, have been recently characterized in many tissues (Doxey *et al.*, 1983). In this study, we characterized the imidazoline receptor in human placenta membranes and attempted to determine its function in trophoblasts. Screening of various tissues for the abundance of IR showed that human placenta is the richest in these sites (1800 ± 100 fmol mg^{-1} protein; K_d = 38,9 ± 3.4 nM). Since human placenta varies from donor to donor, we noticed a heterogeneity in receptor numbers but the most common was around 2000 fmol mg^{-1} protein. The choice of human placenta for studies on IR is also based on the finding that [^{3}H]-idazoxan as well as [^{3}H]-rauwolscine and [^{3}H]-clonidine do not label α_2-adrenoceptors in crude membranes and trophoblast cells. As previously reported by Gradey-Levassort *et al.* (1984), we were not able to identify α_2-adrenoceptors in human placenta.

The pharmacological profile of the placental imidazoline sites is similar to that observed for imidazoline sites in liver and other tissues. A few guanido ligands display a remarkable selectivity for placental imidazoline receptors with high affinity for IR and low affinity for α_2-adrenoceptors (e.g. guanido-pyrol K_i = 11.2 ± 6 nM and > 10 μM respectively). The 40 to 900 fold difference in affinity indicates that the imidazoline site represents a new site with a unique structural requirement. Unlike most other tissues, the placental imidazoline sites display high affinity for amiloride, a Na^+/H^+ antiporter blocker. The difference in affinity of IR for amiloride and its analogue, in different tissues might help in subdividing imidazoline sites into two subtypes: IR_1, which displays high affinity for amiloride (Table 7) and includes sites from human placenta, rabbit kidney, adipocytes and

Table 5 Distribution of imidazoline binding sites (IR), α_2- (α_2 AR) and β-adrenoceptors (βAR) in relation to alkaline phosphatase and Mg-ATPase activities in placental vesicles

	IR	*α_2AR*	*βAR*	*Alkaline phosphatase*	*Mg-ATPase*
	(fmol mg^{-1} protein)			*(μmol min^{-1} mg^{-1} protein)*	*(μgPi min^{-1} mg^{-1} protein)*
Crude membranes	2045 ± 75	0	567 ± 85	141	12.4
Brush-border membranes	233 ± 69	63 ± 30	99 ± 10	628	20.6

Steady state binding of [^{3}H]-idazoxan to IR sites was measured at 4°C after 4 h incubation, binding of [^{3}H]-idazoxan to α_2AR and [^{3}H]-dihydroalprenolol to βAR was determined after 40 min incubation at 30°C. B_{max} values were obtained from Scatchard analysis using the Enzfitter programme.

Table 6 The effect of imidazoline ligands on ATPase activity in placental and brain membranes

Ligand	*Mg^{2+}-ATPase*	*Na^+/K^+ ATPase*
	(μgPi min^{-1} mg^{-1} protein)	
Control	12.9	27.4
Idazoxan	12.5	30.8
Cirazoline	12.9	36.5
Guanabenz	12.1	28.5
Clonidine	12.8	28.5
UK 14304	12.9	—
Naphazoline	12.0	—

Mg-ATPase activity in placental membranes was determined with 150–200 μg protein. The membranes are preincubated in presence of 100 μM ligand for 10 min at 37°C, following the incubation in the presence of 3 mM ATP. The activity obtained in the absence of 5 mM Mg^{2+} was similar to blank value (without enzyme) and was subtracted as a background.
Na/K-ATPase activity in bovine brain membranes (prepared as described by Atlas & Sabol, 1981) was determined using 100–130 μg protein in presence of 100 μM Na^+, 20 μM K^+ and 5 mM Mg^{2+}. The activity was sensitive to ouabain (60% inhibition by 1 mM ouabain).

Table 7 Subdivision of imidazoline receptors (IR) into two subtypes according to their affinity for amiloride

IR subtype	*Tissue*	*Affinity for amiloride K_i (nM)*	*Reference*
IR_a	Human placenta	72	This work
	Rabbit kidney	30	Coupry *et al.* (1989)
	Rabbit fat cells	48	Langin & Lafontan (1989)
	Rabbit liver	160	Tesson *et al.* (1990)
IR_b	Human kidney	1109	Michel & Insel (1989)
	Guinea-pig ileum	2850	Kupitz, Diamant & Atlas unpublished
	Pig kidney	3000	Vigne *et al.* (1989)
	Human fat cells	3873	Langin *et al.* (1990)
	Rat liver	4809	Zonnenschein *et al.* (1990)
	Rat kidney	7760	Michel *et al.* (1989)
	Human liver	10,000	Tesson *et al.* (1990)

liver tissue, as opposed to IR_b, which binds amiloride poorly and is found in a variety of tissues where IR have been described (e.g. rat liver, guinea-pig ileum and rat brain, Table 6). This classification which is based almost entirely on the relative potency of amiloride, is still preliminary and calls for functional responses for confirmation. Similar suggestions were made by Michel & Insel (1989) and Wikberg & Uhlen (1990).

Amiloride competition with [^{3}H]-idazoxan binding to placental membranes, suggests an involvement of imidazoline receptors in Na^+ movement. Indeed in rat kidney cells ^{22}Na influx was inhibited by cirazoline, idazoxan, UK-14304 and rilmenidine, not via α_2-adrenoceptors (Bidet *et al.*, 1990). A comparison of binding constants of amiloride and its analogues with IC_{50} values for Na^+ channel (Cuthbert & Fanelli, 1978), Na^+/H^+ antiporter (Simchowitz & Cragoe, 1986) and Na^+/Ca^{2+} exchangers (Kaczorowski *et al.*, 1985) indicate that IR may be coupled to a Na^+ channel but not to an antiporter or an exchanger. On the other hand, the renal apical Na^+ channel, which was recently cloned, sequenced and expressed in transfected mammalian cells, displays high affinity for benzamil (Barbry *et al.*, 1990), which binds poorly to placental imidazoline receptors ($K_i > 10{,}000$ nM), indicating that IR is also distinct in structure from the amiloride-sensitive Na^+ channel (Table 1). An intact guanido group appears to be essential for recognition at the imidazoline sites. Further studies are needed to verify the possible involvement of Na^+ channel in IR signalling system.

Preparation of placental brush border membranes (defined by enrichment of alkaline phosphatase activity) enabled the localization of IR in assymetrical placental membrane of syncytiotrophoblasts. We found that 90% of IR are located in the basolateral membrane enriched fraction (facing foetal circulation). α_2-Adrenoceptors labelled with [^{3}H]-idazoxan, are absent from the epical-membrane-enriched-fraction but are present in small concentrations in brush border membranes. This finding provides additional distinction between IR and α_2-adrenoceptors according to their anatomical localization. Coupry *et al.* (1989) have also reported asymmetric distribution of IR in the renal proximal tubule.

Recent studies by Gileadi *et al.* (1988) have shown that cytotrophoblasts isolated from term placenta can be grown in monolayers and can be kept for up to 5 days in culture. We found that freshly prepared cytotrophoblasts as well as cells grown in culture (which differentiate to form syncytia *in vitro*) are rich in imidazoline sites. Their cellular location, was evaluated by washing of the extracellularly bound ligand in a low pH/high salt buffer. As shown, these receptors are present on the cell surface and less than 15% of labelled idazoxan is trapped inside the cells. Similar results were published for rat isolated hepatocytes (Zonnenschein *et al.*, 1990).

The trophoblast cells grown in culture were also used to examine possible functions for IR. The unique anatomical and physiological position of placenta between maternal and foetal circulations, determines its function as a mediator in transport of nutrients, ions and oxygen to foetal blood, and waste products from foetal to the maternal circulation. The abundance of IR in tissues such as human placenta, guinea-pig ileum (Kupitz *et al.*, 1991) and rabbit kidney (Coupry *et al.*, 1987) might suggest a role for IR in the mediation of transport.

Since in liver and in placental membranes, binding of [^{3}H]-idazoxan is reduced in the presence of K^+ channel blockers, we postulated that IR might exert effects through a direct interaction with K^+-channel.

However, membrane potential in vesicles derived from brush border and crude membrane of human placenta

(Breuer, 1989) was not affected by any of the guanido or imidazolidine ligands (data not shown). Amino acid transport through human placenta is one of its vital functions. This process is dependent on external Na^+, and Na^+/K^+ ATPase activity serves as its driving force (Shennan & Boyd, 1987). We measured amino acid ([^{3}H]-aminoisobutyric acid) transport into trophoblasts (Johnson & Smith, 1988) assuming that this process may be directly or indirectly (through the effect of Na gating) regulated by the IR receptor. Our preliminary results (not shown) indicate that Na^+-dependent [^{14}C]-aminoisobutyric acid influx into trophoblasts is slightly (up to 40%) affected by guanidine ligands (e.g. guanabenz, guanoxan). However, since the effective concentration was very high (10 to 100 μM), it cast doubt on the aminoisobutyric acid influx being regulated by IR. Further studies are now being undertaken to measure K^+ gating into intact cells with ^{86}RbCl. The transport of other ions and nutrients are also being studied in an attempt to correlate activity to the activation of the IR receptor.

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